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# Warming increased feeding of a root-chewing insect at the soil surface and enhanced its damage on a grass

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ABSTRACT

Air and soil warming influences both plants and root herbivorous insects, but how warming alters their interactions is largely unknown. Because both the intensity of herbivory and plant susceptibility to the herbivory depend on temperature, it is difficult to predict the effects of insect herbivory on plant growth under warming. To analyse changes in belowground plant-insect interaction due to warming, we conducted a pot experiment using one grass species, *Lolium perrene,* and one beetle grub *Anomala cuprea*. Temperature (17 °C or 20.3 °C), presence or absence of a grub, and presence or absence of organic matter (OM) on the surface of the potted soil were manipulated. OM at the soil surface is an important resource for grubs, and contains neutral lipid fatty acids (NLFA) that can serve as a bacterial marker. NLFAs can be used as a tracer to determine whether grubs had fed upon OM, so we evaluated the consumption and vertical movement of a grub in response to warming. In the absence of grubs, plant biomass increased with warming, but was not affected by the OM treatment. In the presence of grubs, plant biomass was significantly decreased. Moreover, the interaction term between the temperature and grub treatments was significant, demonstrating that grub damage was more severe under warmer conditions. Grub mortality was not affected by any treatment. The bacterial NLFAs in a grub were significantly more detectable when OM was added relative to those without OM, and the amount increased with warming treatments. This suggests that the grub fed near the soil surface under both temperature conditions, but increased consumption rates under the warmer condition. The mean relative soil moisture content in the warmer conditions was significantly lower than that in the control conditions. OM additionally increased soil moisture, but it had no effect on herbivory damage, suggesting that plant susceptibility to herbivory was not attributable to soil moisture. These results show the importance of biological-context dependency of warming on a plant. Temperature changes will alter the root-herbivore interactions not by changing the plant susceptibility to herbivory but by increasing the consumption of the grub.

## 1. Introduction

Global mean temperature is predicted to rise by about 3 °C by the end of this century (Meehl et al., 2007). Terrestrial communities and ecosystems are strongly influenced by temperature, which determines geographic species distributions and behaviour of ectotherms. Therefore, understanding the effects of the warming on primary production is crucial for predicting future community dynamics and their services (Millennium Ecosystem Assessment, 2005). Early studies suggested that belowground communities may be buffered from direct impacts of climate change by soil (Bale et al., 2002; Hodkinson and Bird, 1998). However, there is increasing awareness that the interactions between plants and soil fauna determine the response of ecosystem services to environmental changes (Ehrenfeld et al., 2005; Bardgett and Wardle, 2010; van der Putten et al., 2016; Hiltpold et al., 2017).

The magnitude of warming on plant productivity is highly biological-context dependent (Rouifed et al., 2010; Way and Oren, 2010; Jamieson et al., 2012; Makoto et al., 2016) because the warming influences both plants and soil fauna (Bardgett et al., 1999; Frey et al., 2008; van der Putten et al., 2016). Therefore, warming would affect plant productivity directly and indirectly. The direct effects of warming on plants have been intensively studied (e.g., Thomas and Norris, 1981; Zhao and Running, 2010), however, the indirect effects of warming via soil fauna are poorly understood owing to their hidden dynamics

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(Makoto et al., 2014). Especially, roles of belowground insect herbivores are largely unknown, although root herbivorous insects have profound effects on plant growth and mortality (Brown and Gange, 1990; Blossey and Hunt-Joshi, 2003; Tsunoda et al., 2014a) as well as on primary production (Gange and Brown, 2002; De Deyn et al., 2003; Schädler et al., 2004; Stein et al., 2010).

Abiotic conditions such as temperature and soil moisture may alter the behaviour and dietary choice of polyphagous root-chewing insects, the dominant root-feeding guilds such as grubs (Coleoptera: Scarabaeidae), wireworms (Coleoptera: Elatridae), and leatherjackets (Diptera: Tipulidae) (Brown and Gange, 1992; Zvereva and Kozlov, 2012; Johnson et al., 2016). These root-chewing insects can feed on roots of various species, as well as organic matter (OM) derived from leaves. Warming and subsequent changes of soil moisture alter the availability of OM for insects because insects move to deep soil to avoid desiccation of the soil surface (Villani and Wright, 1988, 1990). As a result, the insects cannot reach the OM on the soil surface, and their growth does not increase. Warming directly increases the metabolic rates of soil fauna (Ott et al., 2012). Increasing metabolic rates of soil fauna may lead to increases in their consumption, and consequently their growth. Thus, the effects of warming on the growth of polyphagous root-chewing insects are difficult to predict.

The effects of root herbivory on plants will differ depending on temperature and soil moisture (Villani and Wright, 1990; Erb and Lu, 2013; Tsunoda et al., 2014b). If warming reduces soil moisture, the warming affects plant productivity via the changes in vertical distributions of insects. Because of the filament-like morphology of roots, root herbivory at the root base in the top soil layer often causes plant mortality and large biomass reduction. However, herbivory at root tips does not affect plant performance significantly (Davidson and Roberts, 1969; Tsunoda et al., 2014a, 2014b). Therefore, desiccation of the soil surface caused by warming may reduce the negative effects of root herbivory. However, temperature and soil moisture may also alter the relative value of roots for plant fitness (Rasmann et al., 2011; Erb and Lu, 2013). For example, water limitation can dramatically increase the importance of individual roots: under plentiful amounts of water, plants can tolerate the loss of a significant part of their root system (Erb et al., 2009). However, a relatively minor loss of absorptive tissue can decrease growth when the whole root system is needed to acquire sufficient resources from the soil (Dunn and Frommelt, 1998). Thus, it is hard to predict the effects of warming on interactions between roots and root-feeding insects (Hiltpold et al., 2017).

To understand changes in the interaction due to warming, we conducted a microcosm experiment using a well-studied grass species, *Lolium perenne* L. (Poaceae), and a root-chewing insect, an *Anomala cuprea* Hope (Coleoptera: Scarabaeidae) grub (Tsunoda et al., 2014a, 2014c). Warming increases the growth of *L. perenne* directly (Thomas and Norris, 1981), but its indirect effects via root herbivorous insects have not been examined, as far as we know. We manipulated the air temperature, the presence or absence of the grub, and the presence or absence of OM on the soil surface. Moreover, OM contains some specific fatty acids derived from bacteria, which were used in the experiment as diet markers of the grub (Tsunoda et al., 2017). By analysing the growth and fatty acids of the grub, we evaluated the grub's consumption and vertical movement in response to the warming and subsequent changes in soil moisture. Specifically, we tested how temperature and OM addition influence the behaviour of root-chewing insects, and therefore the effects on the growth of *L. perenne*.

## 2. Materials and methods

### 2.1. Study species

*Lolium perenne* cv. 'amazing' seeds were obtained from a commercial supplier (Snow Brand Seed Co., Ltd, Sapporo, Japan). One *Lolium perenne* L. (Poaceae) seedling was grown in each pot, to which we added a second-instar larva, or grub, of the polyphagous root-chewing insect, *Anomala cuprea* Hope (Coleoptera: Scarabaeidae). The grub feeds on roots of various herbaceous species as well as on OM (Okuno et al., 1978; Sakai and Fujioka, 2007; Tsunoda et al., 2017). Growth of grubs that feed on OM is larger than that of grubs that feed on roots (King, 1977), probably because nitrogen concentration, a limiting factor of the insect growth (van Dam, 2009), is higher in OM than in roots. Grubs were grown from eggs laid by adult *A. cuprea* in the OM (commercial horticulture humus mainly composed of broad leaves, "Kanjuku-fuyohdo", Hirota-shoten Co., Ltd., Tochigi, Japan). The adult *A. cuprea* on *Fallopia japonica* were manually collected from a floodplain of the Tama River (35°38′N, 139°23′E) in June and July 2015. Leaves of *F. japonica* were used as a diet for the adults.

### 2.2. Experimental design

We conducted the experiment from February to May 2016 in growth chambers (Koitotron, Koito Industries, Ltd., Kanagawa, Japan) with a 14-h day/10-h night photoperiod. Plants were grown in 9-cm diameter and 12-cm depth plastic pots, each filled with 500 mL of soil (350–365 g in dry weight). The soil was collected from the experimental garden of Tokyo Metropolitan University (Hachioji, Tokyo: 35°37′N, 139°23′E) in February 2016, and sieved with a 6-mm mesh to eliminate OM and macrofauna. *Lolium perenne* seeds were sown on a tray of peat moss. Ten days after the sowing (27th February), one seedling was transplanted into each pot.

The growth experiment had a three-way randomized factorial design with nine replications. The factors were temperature with two levels (control, 17 °C and warmer, 20.3 °C), presence or absence of OM, and presence or absence of the grub. The OM was sieved to pass through a 6-mm mesh. The control temperature was in the range of soil temperatures of the grub's growing seasons in Tokyo. The temperature increase of 3.3 °C in the warmer conditions represented the predicted increase in air temperature by 2050 in Japan according to Rowlands et al. (2012). All experimental pots received same amount of water during the experiment. Until day 28 after transplantation, 30 mL of water was added every two to three days (i.e., Watered on Monday, Wednesday, and Friday). After day 28, 60 mL of water was added every two to three days because amounts of transpiration increased. We re-randomized the pot arrangement at every watering.

On day 35 after transplantation (2nd April), 150 mL of OM (25–30 g in dry weight) was added on the soil surface. On 12th April, day 10 after OM addition, a weighed grub was introduced to pots of the grub-present treatment. A grub was added to the centre of a pot, and it burrowed into the soil.

On day 85 after transplantation (22nd May, day 40 after introduction of grubs), we harvested plants and divided them into shoots and roots. The shoots and roots were separately dried at 70 °C for three days, and weighed. Plant biomass was defined as the sum of the shoot and root biomass of each plant. At the harvest, we recovered the OM on soil surface for the evaluation of microbial biomass. We recorded the survival of the grubs (Table S1), and weighed a living grub. The surviving grubs were freeze-dried for the fatty acid analysis.

### 2.3. Soil moisture measurement

After the addition of OM, soil moisture was measured with a soil moisture probe ($ECH_2O$, Decagon Devices, Inc., Pullman, Washington, USA). The relative soil-moisture content was measured at top soil layer (one cm of soil) and bottom soil layer (seven cm of soil) every two to three days (i.e., Measured on Monday, Wednesday, and Friday) before the watering. The probe was inserted from a slit in the pot in the top and bottom soil layer. Relative soil-moisture content was calculated as the difference between the measured value and the minimum value during the experimental period, divided by the range between the maximum and minimum values during the measurements (James et al.,

2003).

### 2.4. Fatty acid analysis

#### 2.4.1. Extraction of fatty acids from OM

To evaluate microbial biomass, we extracted phospholipid fatty acids (PLFAs) from the OM following the procedures of Frostegård et al. (1991) and Niwa et al. (2008). In brief, PLFAs were extracted from the freeze-dried samples (ca. 1g) by shaking the material in 135 mL of a single-phase extraction solvent composed of chloroform, methanol, and 0.05M phosphate buffer (pH 7.4) at 1:2:0.8 v/v/v for 2h. The chloroform fraction (which contained FAs) was then separated from the solvent fraction by leaving the solution upright for 20h. We extracted six samples from the pots in which the grub was survived at the harvest in the warmer condition. In the control condition, we extracted from three grub-survived pots and three randomly chosen pots.

#### 2.4.2. Extraction of fatty acids from grubs

The profiles of neutral lipid fatty acids (NLFAs) of the grubs were analysed according to Tsunoda et al. (2017). The total FAs were extracted from a freeze-dried grub in 5 mL of the single-phase extraction solvent with shaking overnight. The solvent was then transferred to a new tube, and the grub was re-extracted with shaking for 3 h in an additional 2.5 mL of the single-phase solvent. Both the solvents were mixed and 0.8 mL of distilled water and 0.8 mL of chloroform were added to the mixed solvent. The solvent was then centrifuged at 1500 rpm for 10 min to separate the solvent phases from the chloroform fraction.

#### 2.4.3. Separating each fatty acid fraction

The chloroform fraction from each sample was transferred to a silica acid column (BOND ELUT LRC-SI; Varian, Palo Alto, CA, USA). The remaining FAs adsorbed in the column were then eluted with 5 mL of chloroform to obtain the NLFAs, with 10 mL of acetone too obtain the glycolipids, and 12 mL of methanol to obtain the PLFA. The NLFA and PLFA fractions were reduced in volume under an $N_2$ flow on a 40 °C block heater.

FAs were methylated following the procedures of Ichihara and Fukubayashi (2010). Methyl nonadecanoate (FA 19:0) was added to each sample as an internal standard. The fatty acid methyl esters were separated by gas chromatography (7890A GC System, Agilent Technologies, Santa Clara, CA, USA) with the Sherlock Microbial Identification System (MIDI, Newark, DE, USA). The fatty acids 14:0iso, 14:0anteiso, 15:0iso, 15:0anteiso, 15:0, 16:0iso, 16:0anteiso, 16:1ω7, 17:0iso, 17:0anteiso, 17:0cyclo, 17:0, 17:0 10methyl, 18:1ω9, 18:1ω5, 18:0 10methyl, and 19:0cycloω8 were used as bacterial markers (Frostegård and Bååth, 1996; Ruess and Chamberlain, 2010; Frostegård et al., 2011).

### 2.5. Statistical analysis

All statistical analyses were performed with R version 3.2.1 (R Core Team, 2015). Homogeneity of variance or normality of the plant biomass and grub fresh weight were tested with on the Bartlett and Shapiro-Wilks tests, respectively. If a grub was not recovered from a pot (see Table S1 for the grub number), the pot was treated as a missing value for the analyses of plant biomass and grub fresh weight.

Relative soil moisture contents were analysed with a generalized linear mixed model (GLMM) assuming a Gaussian error distribution with canonical link function. In this model, the response variable was the relative soil moisture content and the explanatory variables were the temperature, OM, presence or absence of the grub, and interactions between these variables. The random factors were measurement dates and pots. Maximum likelihood estimates of the parameters in the GLMM was determined using the lmer function in the lme4 package (Bates et al., 2015) To determine the effects of the fixed factors, we used a Wald test and the chi-squared test statistic in the car package (Fox and Weisberg, 2011). Relative soil moisture content was arcsine-transformed (Sokal and Rohlf, 1995) and analysed by Tukey's honestly significant differences (HSD) test.

The effects of the treatment combinations on plant biomass were analysed by three-way analysis of variance (ANOVA). The plant biomass was treated as the response variable. The explanatory variables were the temperature, OM, presence or absence of a grub, and interactions between these variables. The plant biomass was also analysed by Tukey's HSD test.

The grub mortality was analysed with a GLM assuming a binomial error distribution with a canonical link function. In this model, the response variable was the grub mortality and the explanatory variables were the temperature, OM, and interaction between these variables. To determine the effects of the fixed factors, we used a Wald test and the chi-squared test statistic in the car package (Fox and Weisberg, 2011).

The effects of the treatment combinations on grub fresh weight were analysed by two-way analysis of covariance (ANCOVA). The grub fresh weight at the harvest was treated as the response variable and grub fresh weight at the beginning of the experiment was the covariate. The explanatory variables were the temperature, OM and their interaction.

Non-metric multidimensional scaling (NMDS) was conducted by calculating the Chao dissimilarity index (Chao et al., 2005) to detect effects of the experimental treatments on the overall grub NLFA structure. The NMDS was performed using the metaMDS function of the vegan package of R (version 2.3–0; Oksanen et al., 2015). We tested for significant differences in the NMDS axis scores using permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001; McArdle and Anderson, 2001). In the PERMANOVA model, the content of each NLFA was treated as the response variable. The explanatory variables were the temperature, OM, and their interaction.

The concentrations of sum of the bacterial marker NLFA in the grubs were analysed by two-way ANOVA. The concentration was treated as the response variable, and the explanatory variable was the warming and OM. Significant differences between pairs of means were tested by means of Tukey's HSD test. In all cases, significance was set at $P < 0.05$. Because very many NLFA profiles were detected, only bacterial NLFAs were reported in the main text. All NLFAs are shown in the supporting information (Figs. S1 & S2).

## 3. Results

### 3.1. Relative soil-moisture

The mean relative soil-moisture content in warmer conditions was significantly lower than those in the control temperature (Fig. 1; Table 1). The relative soil-moisture content of the top soil layer was significantly lower than that of the bottom soil layer. Moreover, the main term of the OM treatment and the interaction term between the OM treatment and soil layer were significant on the relative soil-moisture content, which mean the relative soil-moisture content with the OM conditions was significantly higher than that without OM in the top soil layer but did not differ significantly in the bottom layer.

### 3.2. Plant biomass

ANOVA revealed that the grub main effect and the interaction term between the temperature and presence of the grub were significant (Table 2). The reduction of plant biomass in the grub present condition was larger under the warmer conditions than under the control temperature (Fig. 2).

### 3.3. Grub performances

Grub mortality did not significantly change by the warming and OM treatments (Tables S1 & S2). Two-way ANCOVA revealed that the grub

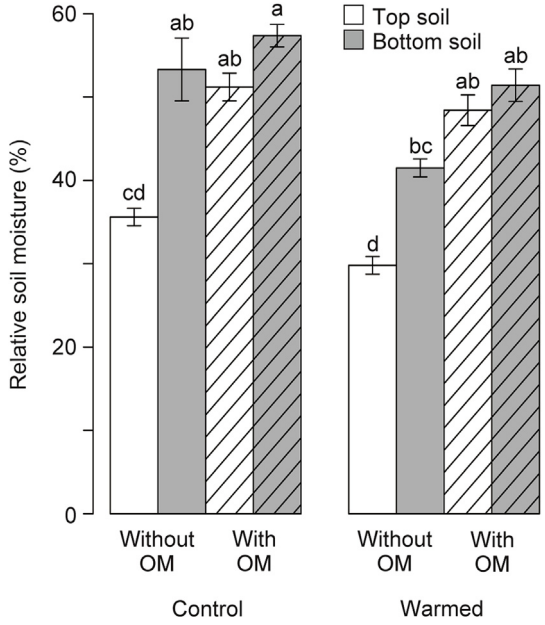


**Fig. 1.** Mean relative soil moisture content (% ± SE). Open bars, without the organic matter (OM): Hatched bars, with the OM: White bars, top layer of soil: Grey bars, bottom layer of soil. Differences between bars with different letters are significant ($P < 0.05$, Tukey's HSD post-hoc test).

**Table 1**
The Wald test to test effects of temperature warming (T), addition of the organic matter (O), and soil layer (L) on relative soil water content.

| | df | $\chi^2$ | *P* |
|---|---|---|---|
| T | 1 | 22.407 | < **0.001** |
| O | 1 | 74.864 | < **0.001** |
| L | 1 | 60.450 | < **0.001** |
| T × O | 1 | 2.542 | 0.111 |
| T × L | 1 | 3.427 | 0.064 |
| O × L | 1 | 16.641 | < **0.001** |
| T × O × L | 1 | 0.330 | 0.566 |

Bold indicates significant difference P-values (P < 0.05).

**Table 2**
Analysis of variance to test effects of temperature warming (T), addition of the organic matter (O), and presence or absence of the grub (G) on *Lolium perenne* biomass.

| | df | SS | *F* | *P* |
|---|---|---|---|---|
| T | 1 | 0.045 | 0.972 | 0.329 |
| O | 1 | 0.012 | 0.253 | 0.617 |
| G | 1 | 3.636 | 78.734 | < **0.001** |
| T × O | 1 | 0.000 | 0.001 | 0.972 |
| T × G | 1 | 0.334 | 7.237 | **0.010** |
| O × G | 1 | 0.001 | 0.016 | 0.901 |
| T × O × G | 1 | 0.000 | 0.004 | 0.948 |
| Residuals | 49 | 2.263 | | |

Bold indicates significant difference P-values (P < 0.05).

fresh weight at the beginning of the experiment was marginally correlated with that after the experiment (Fig. 3; Table 3). The correlation of grub fresh weight between the beginning and the end of the experiment differed significantly between the OM addition. When OM was added, grubs grew larger. Warming did not significantly affect the grub fresh weight.

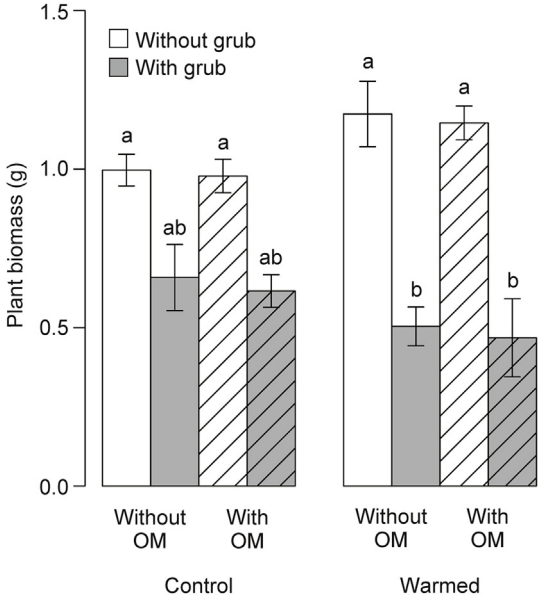


**Fig. 2.** Mean plant biomass (g ± SE). Open bars, without the organic matter (OM): Hatched bars, with the OM: White bars, without the grub: Grey bars, with the grub. Differences between bars with different letters are significant ($P < 0.05$, Tukey's HSD post-hoc test).

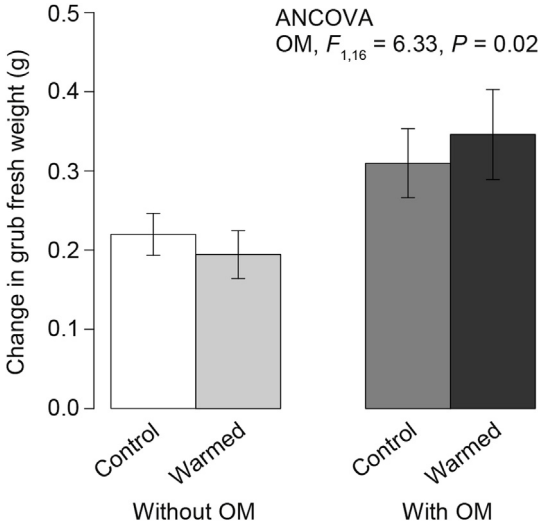


**Fig. 3.** Relationships between the grub's fresh weight before and after the experiment for the four combinations of the warming and addition of the organic matter (OM).

**Table 3**
Analysis of covariance to test effects of the temperature warming (T) and addition of organic matter (O) on the grub fresh weight after the experiment. Grub fresh weight at the beginning of the experiment (B) was treated as a covariance.

| | df | SS | *F* | *P* |
|---|---|---|---|---|
| B | 1 | 0.042 | 4.412 | 0.052 |
| T | 1 | 0.000 | 0.039 | 0.847 |
| O | 1 | 0.060 | 6.330 | **0.023** |
| T × O | 1 | 0.007 | 0.709 | 0.412 |
| Residuals | 16 | 0.151 | | |

Bold indicates significant difference P-values (P < 0.05).

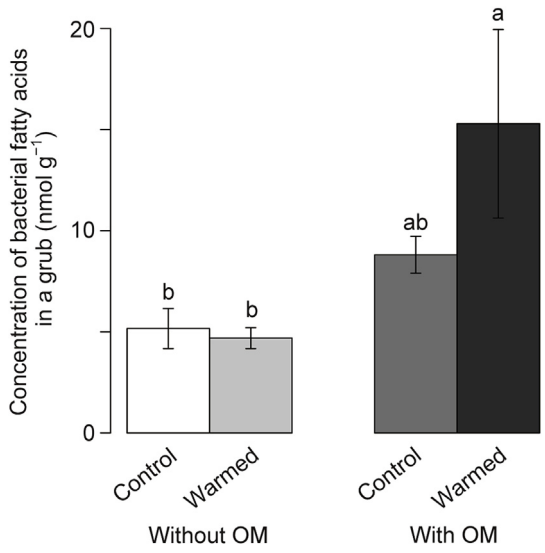


**Fig. 4.** Mean concentration of bacterial neutral lipid fatty acids in a grub (nmol $g^{-1}$ ± SE). White, control temperature; light grey, warmer temperature; dark grey, control temperature with the organic matter (OM); black, warmer temperature with OM. Differences between bars with different letters are significant ($P < 0.05$, Tukey's HSD post-hoc test).

**Table 4**
Analysis of variance to test the effects of the temperature warming (T) and organic matter (O) on the bacterial neutral lipid fatty acids in a grub.

| | df | SS | *F* | *P* |
|---|---|---|---|---|
| T | 1 | 0.040 | 0.156 | 0.698 |
| O | 1 | 3.328 | 12.898 | **0.002** |
| T × O | 1 | 0.164 | 0.637 | 0.436 |
| Residuals | 17 | 4.386 | | |

Bold indicates significant difference P-values (P < 0.05).

### 3.4. PLFAs of the OM and NLFAs of the grub

The concentration of bacterial PLFAs of the OM did not differ from temperature conditions (Fig. S3; *t*-test, $P = 0.926$). The concentration of bacterial NLFAs in a grub grown with OM were significantly higher than that in a grub grown without OM (Fig. 4; Table 4). The concentration of bacterial fatty acids in a grub was significantly higher under the warming and OM condition than under the no OM conditions (Tukey's HSD). The NLFA profiles of a grub significantly differed in the OM addition but did not differ by the warming (Figs. S2; Table S3).

## 4. Discussion

Our microcosm experiment revealed that warming enhances the negative effect of grub herbivory on the biomass of *L. perenne*. These results clearly show biological-context dependent effects of warming on plant productivity (Rouifed et al., 2010; Way and Oren, 2010; Jamieson et al., 2012; Makoto et al., 2016). Warming altered the effects of root herbivorous insects on plant productivity. Here, we discuss the mechanism how warming enhanced herbivory damage focusing on the effects of warming on both insect feeding behaviour and plant susceptibility to herbivory.

As we expected, the OM addition enhanced grub growth and increased bacterial NLFAs in a grub. These results are consistent with previous studies (King, 1977; Tsunoda et al., 2017). However, warming did not decrease bacterial NLFAs in a grub or grub fresh weight, which suggests the grub fed on the OM in the top soil layer in both control and warmer conditions. Because soil moisture is one of the most influential factors on belowground insect behaviour (Villani and Wright, 1988, 1990; Brown and Gange, 1990), grubs could dig themselves into deeper soil to avoid the moisture loss of the soil surface. However, there was no evidence to support the vertical movement of the grub. Thus, the tested warming and changes in soil moisture had no significant effects on the vertical distributions of the grub.

The amount of OM consumed by the grub could not be measured because the grub mixed OM with soil, thus OM was difficult to recover. However, the result of bacterial NLFAs in a grub suggests that warming increased consumption of OM. This is consistent with a previous study that warming directly increases metabolic rates of soil fauna (Ott et al., 2012). The increased consumption of the grub increases the amount of detached roots and thus increase damage to the plant. We cannot evaluate amounts of consumed roots, but grubs would feed on both OM and roots because intake of both diets increases growth of grubs.

In the present study, soil was significantly drier in conditions without OM than those with OM. If the desiccation increases plant susceptibility to herbivory, herbivory damage is greater in conditions without OM than in those with OM because the whole root system is needed to acquire sufficient resources from the soil under stressed environments (Dunn and Frommelt, 1998). However, in the present study, herbivory damage did not differ depending on the OM conditions, which suggests the decreased soil moisture did not affect plant susceptibility to herbivory.

In conclusion, the predicted warming will alter the plant-insect interaction by increasing the feeding of the grub on plants and OM. In Thomas and Norris (1981), warming increased *L. perenne* growth because warming increased leaf turnover and leaf area index. These results suggest that warming increase metabolic rates of *L. perenne*, which affected the biomass gain. In the present study, warming increased the biomass of *L. perenne* under conditions without a grub, too. However, warming affects metabolic rates of soil fauna as well (Ott et al., 2012). Indeed, bacterial NLFAs in a grub remarkably increased under the warming condition. As a result, warming increased the biomass reduction due to root herbivory under the warmed conditions. Early studies suggested that belowground communities may be buffered from direct impacts of climate change by soil (Bale et al., 2002; Hodkinson and Bird, 1998), warming increase grub consumption and change primary production.

## Authorship

T.T. and K.M. designed the study. T.T. carried out the experiments, analysed data and wrote the first draft of the manuscript. All authors contributed to writing this article.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.soilbio.2018.09.009.

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